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Novel solid-state fermentation extraction of 5-*O*-caffeoylquinic acid from heilong48 soybean using *Lactobacillus helviticus*: Parametric screening and optimization

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ABSTRACT

This study investigated the extraction of 5-*O*-caffeoylquinic acid (5-CQA) with increased yield and enhanced antioxidant activity from heilong48 soybean (HS) under solid-state fermentation (SSF). Plackett–Burman design and Box-Behnken design were sequentially used for screening and optimization of significant SSF conditions respectively. Screening results showed that temperature, pH, incubation time and liquid-solid ratio were the significant SSF conditions that influenced 5-CQA yield, fermentation efficiency and antioxidant activity. The optimum SSF conditions obtained by Box-Behnken design were 49.90 °C (temperature), 7.00 (pH), 25.81 h (incubation time) and 0.67 (liquid-solid ratio). For these conditions, the experimental data obtained [5-CQA yield (11.41 ± 0.27 mg/g), fermentation efficiency (30.49 ± 1.14%), and antioxidant activity (46.13 ± 1.94 μmol AA eq/g dry sample)] were consistent with predicted values, higher than that of unfermented HS flour (RSHF), and supported by Atomic force microscopy (AFM), Fourier transform infrared (FTIR) and Scanning electron microscopy (SEM) microstructure. The results demonstrated that optimized SSF conditions significantly influenced 5-CQA yield, fermentation efficiency and antioxidant activity. This study showed that the use of optimized SSF conditions to extract 5-CQA with increased yield and enhanced antioxidant activity was efficient. Hence, this could be useful to the food and/or pharmaceutical industry in producing 5-CQA from HS.

1. Introduction

The concept of extracting and processing plant bioactive components into useful substances/products for human utilization is profitably agreeable. Hence additional research in the fields of food science and engineering, biotechnology and nanotechnology, on this subject, is worth looking at (Verduzco-Oliva & Gutierrez-Uribe, 2020). One most important technology in the mentioned areas/fields that cannot be left out if bioactive ingredients of plant are to be harnessed for human benefits is fermentation. Fermentation is an ancient biotechnology and classic industrial process for improving the shelf-life, nutritional and organoleptic qualities of food (Magro, Silva, Rasera, & de Castro, 2019). It also increases the release of biologically active compounds having antidiabetic and antioxidative activities (Magro et al., 2019).

Solid-state fermentation (SSF), a type of fermentation, is a cost-effective and green technique with much attention received for its processing and biological advantages relative to submerged and liquid fermentation (Ang, Ngoh, & Chua, 2013). SSF, alternative to submerged fermentation (with greater advantages than submerged fermentation in various processes) is widely used for production of products with added values namely enzymes, single cell protein, antibiotics, poly-unsaturated fatty acids, organic acids, aroma, biofuel and bio-pesticides (Bhargav, Panda, Ali, & Javed, 2005). SSF has numerous biotechnological benefits such as higher product stability, reduced catabolic repression, higher fermentation or volumetric productivity, lower demand on sterility, less effluent generation, higher concentration of end-products, use of water-insoluble substrates specific microorganisms, simple fermentation equipment requirement (Kapilan, 2015) as

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well as environmentally friendly and more cost- and energy-effective (Sitanggang, Sinaga, Wie, Fernando, & Krusong, 2020). It is therefore a promising bioconversion technology for pant products valorization into high value-added products.

Fermentation in previous years was done to increase bioactive phenolic compounds content in legumes, consequently improving their antioxidant activity (Bartkiene, Krungleviciute, Juodeikiene, Vidmantiene, & Maknickiene, 2015). This bioprocess has been researched as an efficient method for the extraction and production of biologically active compounds in food lately (Handa et al., 2019). Soybean (valuable legume worldwide) is a high nutritional, economic and suitable substrate for SSF utilization for numerous applications to produce value-added foods and antioxidant compounds (Correa Deza, Rodríguez de Olmos, & Garro, 2019). Hu et al. (2019) reported that fermentation of soybeans decreases antinutritional factors, lipoxygenase, urease and trypsin inhibitor activities. Products of fermented soybean are high in antioxidative activities (Yang et al., 2019), and more attention given to those with high nutrition and health benefits (Bartkiene et al., 2015). The products obtained after whole soybean SSF are directly lyophilized with no centrifugation. Thus, SSF of whole soybean is a more cost-effective, simple technology for probiotics carrier food production. Soybean has been fermented to produce specific foods that contain phenolic antioxidants with related consumer good health and wellbeing (Handa et al., 2019).

One important phenolic acid with many health benefits, obtained from soybean, but least investigated is 5-*O*-caffeoylquinic acid (5-CQA) (Nabavi et al., 2017; Naveed et al., 2018). 5-CQA has tremendous application in food, pharmaceutical and cosmetic industries. It has been the focus of interest due to its putative health benefits and impact on food quality. Due to the numerous health benefits of 5-CQA, its demand is on the rise; however, the cost of production is high, limiting its availability for human benefits. The reason for this is that, existing works on 5-CQA production/extraction focused on using coffee (an expensive cash crop which is not available all-year round) as the raw material. Also, conventional methods that use organic solvents (e.g. chloroform, dichloromethane, etc.) are commonly used to extract 5-CQA. These solvents, however, are dangerous to handle and harmful to human health and the environment (Torres-Mancera et al., 2013). In addition, the conventional methods (that make use of dichloromethane, methanol, ethanol, acetone extraction, etc.) in extracting 5-CQA are: time-consuming, relatively high in solvent usage, often unsatisfactory in reproducibility and poor in the extraction of polar substances (Wianowska & Gil, 2019). As a result, the food, pharmaceutical, and cosmetic industries are lately searching for rich and cost-effective plant sources (for 5-CQA) and also efficient extraction techniques (Wianowska & Gil, 2019). That notwithstanding, existing research on soybeans (a cheap crop available all-year round) are limited to isoflavones, even with the new varieties with improved qualities. Till date, the only literature on soybean in connection with 5-CQA dates back to 1979, where it was only reported as a source of 5-CQA (Pratt & Birac, 1979) with no further work/data on content quantification or extraction.

SSF (most efficient bioprocess) could be an efficient approach to increase the release of 5-CQA from soybean. SSF enhances the phenolic content in plant extracts via the breakage of ester bonds between the plant cell wall and phenolics, increasing their concentration and hence functional properties (Santos da Silveira et al., 2019). Due to the low water availability in SSF, a limited number of microorganisms are used. Filamentous fungi are considered the most desirable microbes for SSF followed by yeasts and moulds (Santos da Silveira et al., 2019). Though, in nature, filamentous fungi and bacteria typically grow on solid substrates (leaves, roots, seeds, stems and wood of plants) in symbiotic associations (Kapilan, 2015), bacteria are not considered for SSF. Some bacteria species (*Bacillus thuringiensis*, *Bacillus subtilis* and *Lactobacillus* sp.), however, have been reported for SSF (Soccol et al., 2017). Lactic acid bacteria have been extensively utilized in soybean fermentation to produce soybean flour, sufu (Chinese soy-food), and soybean-milk (Zhang et al., 2014). However, using lactic acid bacteria for extraction of 5-CQA from soybean under SSF has not been investigated. As a result, the present study sought to screen and optimize SSF parameters for extraction of 5-CQA with increased yield and improved antioxidative activity from heilong48 soybean (HS) variety using *L. helviticus* strain. Plackett–Burman design and Box-Behnken design were sequentially used to achieve the study objective. Plackett–Burman design was used for the screening of the SSF conditions to identify the significant SSF conditions that influence 5-CQA extraction and Box-Behnken design used for the optimization of the significant SSF conditions obtained for extraction of 5-CQA with increased yield and enhanced antioxidant activity. Second-order polynomial model was developed for the extraction process and evaluated using analysis of variance for the model credibility. Structural analyses [Atomic force microscopy (AFM), Fourier transform infrared (FTIR) and Scanning electron microscopy (SEM)] were performed to affirm the effectiveness of the optimized SSF conditions on the degradation of the cell wall of heilong48 soybean (HS) variety to release more 5-CQA.

2. Materials and methods

HS variety was purchased from Tianxia Agricultural and Sideline Products and Distribution Department, China. *L. helviticus* LH-43 was bought from Synbio Tech Inc., Taiwan. It was stored at 4 °C until use. Only analytical grade chemicals were used in this study.

2.1. HS flour preparation

HS variety was milled with a hammer crusher (FC160, Shanghai traditional Chinese medicine machinery factory, China) and further sieved into fine flour of particle size 0.25 mm. The final flour was packed in air-tight low density polyethene bags in weights of 150 g and stored (−20 °C) for further studies.

2.2. Inoculum preparation

L. helviticus LH-43 was activated by subculturing twice in de Man, Rogosa and Sharpe (MRS) broth at 37 °C for 24 h (Zhou et al., 2019). The culture was centrifuged using RJ-TDL-50A centrifuge (Ruijiang Analytical Instrument Co., Ltd., China) at 4000×*g* for 10 min. The supernatant was discarded and the bacterium cells washed in sterile saline (0.1% NaCl) solution. An XB-K-250 hemocytometer (Jianling Medical Device Co., China) was used to measure the inoculum concentration and corrected to 10^9 CFU/ml with 0.1% sterile NaCl solution. The obtained suspension was used as starter culture for SSF.

2.3. SSF of HS variety

Sterile distilled water was added to 10 g HS flour (on dry matter basis) to attain different moisture contents (20, 30 and 40%) in a conical flask (250 ml). The contents were thoroughly mixed and sterilized for 15 min at 121 °C (Li et al., 2020). After cooling to 25±2 °C, the mixture was inoculated with 1, 3 and 5% inoculum of *L. helviticus* with 10^9 CFU/g cell population. This was followed by thorough mixing and culturing at different temperatures (30, 40 and 50 °C) in an incubator (SPX-250, Jintanshizhongdayiqichang, China) for 0, 24 and 48 h under static aerobic conditions. The pH of the SSF of HS variety was adjusted through the addition of the 1 N NaOH or 1 N HCl to the culturing medium (Adnan, Ashraf, Khan, Alshammari, & Awadelkareem, 2017). All fermented samples were stored at −20 °C for further investigations.

2.4. Screening with Plackett-Burman design

Plackett–Burman design was utilized to screen the significant factors (having influence on SSF process for 5-CQA extraction with increased yield) – temperature (Temp), pH, incubation time (IT), inoculation size

(IS) and liquid-solid (L-S) ratio coded A, B, C, D and E respectively, in ranges of 30–50 °C, 5–7, 0–48 h, 1–5%, and 0.25–0.67. Two factorial (−1 and +1) design locating significant variables for the extraction by screening "n" variables in "n+1" experiments was used. Thirteen runs of different combinations of independent variables A – E given by the design were investigated at high (+), mid (0) and low (−) levels. Plackett–Burman design was achieved based on a first-order polynomial model (Boateng, Yang, & Li, 2020):

$$Y = \beta_0 + \sum_{i=1}^{5} \beta_i X_i \quad (1)$$

where; Y = response, β_0 = model intercept and β_i = linear coefficients, X_i = independent variables.

2.5. Optimization with Box-Behnken design

The four significant variables; temperature (X_1), pH (X_2), incubation time (X_3) and liquid-solid ratio (X_4) selected from Plackett–Burman design experiment were further subjected to Box-Behnken design (optimization) analysis to increase the yield of 5-CQA from HS variety. A 4-factor-3-level Box-Behnken design comprised of 29 experimental runs was used. A second-order polynomial model was fitted to correlate the association of each parameter to the response. The equation as adopted by Wang et al. (2020) was used:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{3} \times \sum_{j=i+1}^{3} \beta_{ij} X_i X_j \quad (2)$$

where; Y = predicted dependent variable, β_0 = intercepts, $\beta_{i,}$ = linear regression coefficients, $\beta_{ii,}$ = second-order regression coefficients and β_{ij} = interaction regression coefficients, all estimated by the model. X_i and X_j = independent factors. Overall Desirability Index (DI) was used to select the optimized parameters from the equation (Akpabli-Tsigbe et al., 2021) below:

$$DI = \left[\prod_{i=1}^{3} d_i(y_i) \right]^{1/3} \quad (3)$$

where; d_i = Desirability Index of response variable (0–1) and y_i = responses.

2.6. Standard 5-CQA solution preparation

For the standard 5-CQA solutions preparation, a commercial 5-CQA (MO 63103, Sigma-Aldrich, Co., USA) was used. The method of Adane, Yoseph, and Kusse (2019) with slight modification was used for the preparation of the standard solution. 1000 mg was dissolved in 1-L distilled water to prepare stock standard 5-CQA solution. The solution was uniformly mixed using magnetic stirrer (C-MAG HS 7 S025, IKA, Germany) in the dark. Series of standard solutions (5, 10, 15, 20, 25 and 30) mgL^{-1} were prepared from the stock solution for 5-CQA in distilled water. All measurements were done within 10 min after preparation and absorbance of each series of standard 5-CQA solutions was taken immediately. The method was validated against Beer-Lambert's law with the series of standard 5-CQA solutions prepared.

2.7. Antioxidant activity determination

The antioxidant activity of the raw (unfermented) HS flour (RHSF) and *L. helviticus* fermented HS (LHFHS) was determined by 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity using the method described by Haida and Hakiman (2019) with slight modification. Aliquots of 1 ml RHSF and LHFHS extracts were added to 2 ml of 1 mM methanolic dilution of 2,2-diphenyl-1-picrylhydrazyl (1×10^{-3} M). The mixture after vortexing, was incubated in the dark for 30 min at 37 °C and absorbance taken at 517 nm against a blank in a UV-1600 spectrophotometer (Beijing Rayleigh analytical instrument, China). The results were expressed in micromoles ascorbic acid equivalents per gram of dry sample (μmol AA eq/g dry sample) using ascorbic acid standard curve generated under same conditions. The linear range for ascorbic acid standard was 12.50–800.00 μg/ml ($r^2 = 1.00$).

2.8. Total phenolic acids determination

Total phenolic acids were determined by adopting the method known as the Folin–Ciocalteu phenol reagent technique reported by Haida and Hakiman (2019), with minor modification. Briefly, 1 ml each of HS and LHFHS extracts was added to 9 ml of distilled water in separate test tubes. Then, 1 ml of Folin–Ciocalteu phenol reagent was added to it and the mixture was mixed thoroughly via vortex. After 5 min, 10 ml of 7% sodium carbonate was added. Next, 4 ml of distilled water was added and the mixture was adjusted to 25 ml of final volume. The reaction mixture was incubated for 90 min at room temperature, and the absorbance was measured at 750 nm in a UV-1600 spectrophotometer (Beijing Rayleigh analytical instrument, China). The total phenolic acids were expressed as milligram of gallic acid equivalents per gram of sample (mg GAE/g sample). A standard curve for gallic acid (as standard) in methanol was prepared using different concentrations (100–700 μg/ml).

2.9. Determination of 5-CQA

5-CQA determination was performed according to advanced procedures from previous studies (Adane et al., 2019) with slight modification. 40 mg amount of RHSF and LHFHS samples was weighed and dissolved in 30 ml distilled water in a 100 ml beaker separately. The solution was stirred for 30 min using magnetic stirrer (model C-MAG HS 7 S025, IKA, Germany) and heated (at 40 °C) to increase the solubility of 5-CQA in solution. The solution was filtered through double-loop qualitative filter paper (NO. 1568, Ge Biotechnology Co., Ltd, China) to get rid of particles from solution. The filtrate containing 5-CQA was collected and measured to obtain volume of the sample extract. The absorbance of the measured sample extract was taken using UV-1601 spectrophotometer (Beijing Rayleigh Analytical Instrument Co. Ltd, China) within wavelength ranges of 190–1100 nm from which 5-CQA concentration was computed against the standard solution by Beer Lambert's Law at the maximum wavelength (λ_{max} = 325 nm). Equations (4) and (5) as adopted by Adane et al. (2019) were used to compute 5-CQA content and % 5-CQA of RHSF and LHFHS respectively:

$$5-CQA\ content\ (mg) = \frac{[5-CQA\ conc\ (mg/L)] \times [total\ sample\ volume\ (ml)]^2}{[measured\ sample\ volume\ (ml)] \times 1000} \quad (4)$$

$$\%\ 5-CQA\ (w/w\%) = \frac{[calculated\ mass\ of\ 5-CQA\ (mg)]}{[mass\ of\ sample\ measured\ (mg)]} \times 100\% \quad (5)$$

2.10. Fourier transform infrared (FTIR) analysis

FTIR spectroscopy was applied to examine the structure of LHFHS and RHSF samples according to the method described in the literature (Musa et al., 2019) with slight modification. Briefly, 1 mg from the freeze-dried fermented and raw (unfermented) HS powder (control) was thoroughly mixed and ground with 200 mg of dried spectroscopic grade KBr (at 105 °C for 24 h) powder separately in a mortar with pestle (both made of agate). The resulting mixture was compacted with hydraulic machine (15 t) into a see-through (transparent) glass-like pellets of thickness, 1–2 mm. The pellets were scanned in the wavenumber ranging from 4000 to 400 cm^{-1} with 128 scans using model Nicolet IS50 device (Thermo Nicolet Corporation, USA) at a resolution of 4 cm^{-1}. The blank (KBr pellet without test samples) used under setting parameters

was reported as reference spectra.

2.11. Scanning electron microscopy (SEM) analysis

The structure of LCFHS and RHSF samples was examined using SEM method described by Musa et al. (2019) with slight modification. The LHFHS and RHSF samples were placed on a copper sample-holder with double-sided adhesive tapes and coated with a conductive layer of gold powder (about 10 nm) by using vacuum coating apparatus. Their structures were examined with Hitachi S–3400N (Hitachi High Technologies, Tokyo, Japan) at 15 kV acceleration voltage.

2.12. Atomic force microscopy (AFM) analysis

The method outlined by Dabbour et al. (2020) (slightly modified) was used to determine the topography of LCFHS and RHSF samples. Dissolution of LHFHS and RHSF samples were done in 0.01 M saline phosphate buffer (pH 8.0) to prepare 10 μg/ml final concentration. The solution was heated in thermostatic water bath (50 °C) for 10 min and centrifuged (4000 rpm, 10 min). 5 μL aliquots of the supernatant were rapidly pipetted onto a newly cleaved mica substrates, placed in petri dishes and dried in an incubator (25 °C) for 12 h. Multimode microscope (Bruker, Santa Barbara, CA) was used to generate the AFM images of the samples. The lens was used in Peak Force^{QNM} mode with Bruker ScanAsyst needle at a typical spring and resonance frequency of 25.1 N/m and 300 kHz respectively.

2.13. Statistical analysis

Version 11.0.5.0 Design Expert Software (STAT-EASE, Inc., USA) was used for the experimental designs and optimization. MINITAB v18.1 software (Minitab Inc., USA) was used to screen the variables. Accuracy of the model was evaluated with P-test, determination coefficient (R^2), lack of fit test and variation coefficient (CV), represented at $p < 0.05$, 0.01 and 0.001. All experiments were done three times and data processed with MS Excel 2016 (Microsoft Corporation, USA). All graphs were constructed using OriginPro version 2018 (OriginLab Corporation, USA). All values were reported as mean ± standard deviation. Tukeys' test was used for comparison of the means at $p < 0.05$.

3. Results and discussions

In this study, a chronological optimization plan involving two stages/phases was used. The first phase involved screening of various SSF conditions and identifying those with the significant effects on critical variables affecting 5-CQA extraction from HS variety using *L. helviticus*. The comparative importance of the various SSF conditions was studied with Plackett-Burman experimental design. Once these significant conditions were determined, the second phase ascertained their combinations for best useful SSF conditions for 5-CQA extraction with increased yield. Response surface methodology, a mathematics method based on the suitability of polynomial (quadratic) equation to an experimental data (Bezerra, Santelli, Oliveira, Villar, & Escaleira, 2008) was applied to achieve this aim.

3.1. Influence of SSF parameters on 5-CQA extraction

From Electronic Supplementary Table 1c, the *F*-values for 5-CQA yield, fermentation efficiency, total phenolic acids and antioxidant activity obtained were 18.11, 17.20, 2.80 and 20.47 respectively, indicative of significant model. However, the *F*-value for total phenolic acids was relatively small and probably suggested that it was not an important indicator for evaluating the effects of the SSF conditions for 5-CQA extraction from HS variety. The model for total phenolic acids was therefore not used for predictive purposes due to its low R^2 value (29.77%). According to Handa et al. (2019), the R^2 value of an appropriate proposed model should be close to 100% since it indicates a better explanation of the variability of the experimental data by the proposed model. And also shows that a better correlation exists between observed and predicted values (Mintah et al., 2020). The effects of the SSF parameters on the responses and the statistical significance were shown by Pareto chart (Fig. 1). This study obtained 2 limit lines namely Bonferroni limit line (2.733) and *t*-value limit line (2.035). The *t*-values of effect above the Bonferroni limit line were considered extremely significant, between Bonferroni limit line and *t*-value limit line were considered significant and below *t*-value limit line were considered non-significant (Guo et al., 2018). These determinants were used to determine the extremely significant SSF parameters for 5-CQA extraction from HS variety. The *t*-value effect of temperature and pH were above the Bonferroni limit line and positive for 5-CQA yield, fermentation efficiency and antioxidant activity, suggestive that increasing of temperature and pH increased 5-CQA yield, fermentation efficiency and antioxidant activity. Likewise, incubation time had a positive *t*-value of effect (above the Bonferroni limit line) for 5-CQA yield and fermentation efficiency. However, liquid-solid ratio exhibited a negative *t*-value effect (above the Bonferroni limit line) for all responses (5-CQA yield, fermentation efficiency and antioxidant activity) indicative that increasing of liquid-solid ratio decreased 5-CQA yield, fermentation efficiency and antioxidant activity.

The decrease of antioxidant activity with increased liquid-solid ratio was due to oxidation or degradation of antioxidant compounds (Kaprasob, Kerdchoechuen, Laohakunjit, Sarkar, & Shetty, 2017) dependent on the synergetic and redox interactions among the different compounds in HS, thus led to the low antioxidant activity of LHFHS sample. Similarly, the decrease of the 5-CQA yield at increased liquid-solid ratio was due to degradation of 5-CQA (Heo, Adhikari, Choi, & Lee, 2020). Only the *t*-value effect of pH was above the *t*-value limit line for total phenolic acids. All the SSF parameters except inoculation size were extremely significant (above Bonferroni limit-line) for 5-CQA yield and fermentation efficiency. Temperature, liquid-solid ratio and pH were extremely significant for antioxidant activity. The *t*-value of effect of inoculation size was below the *t*-value limit line for all the responses. The results revealed that temperature, pH and incubation time showed significant positive effect on 5-CQA extraction from HS variety with the exception of liquid-solid ratio which exhibited a significant negative effect (Fig. 1a). This implied that increasing temperature, pH and incubation time, increased 5-CQA extraction from HS variety while the reverse was obtained for liquid-solid ratio; increasing liquid-solid ratio, decreased 5-CQA extraction. The effect of inoculation size on 5-CQA extraction, however, was not significant though negative, suggestive that the effect of inoculation size (increasing or decreasing) on 5-CQA extraction was negligible. This showed that the four SSF parameters (temperature, pH, incubation time and liquid-solid ratio) were the most significant factors for 5-CQA extraction from HS variety, hence selected and used in the following experiments.

Though inoculation size increased, it's *t*-value of effect was not significantly enough as shown by the interaction effect of Plackett–Burman design matrix. Likewise, the influence of inoculation size on the various responses was insignificant. Nonetheless, inoculation size (the non-significant SSF parameter) was also studied in initial experiments. The results revealed that an average inoculation size of *L. helviticus* was adequate for maximum growth, proliferation and initially colonization of most HS substrate without overcrowding and competition for nutrient, resulting in high fermentation efficiency and 5-CQA extraction from HS variety. Inoculation size of 3% was therefore used in further experiments which was slightly lower than that (4%) used by Gao, Wang, Zhu, and Qian (2013) for *A. oryzae*. This could be due to the differences in the microbial types.

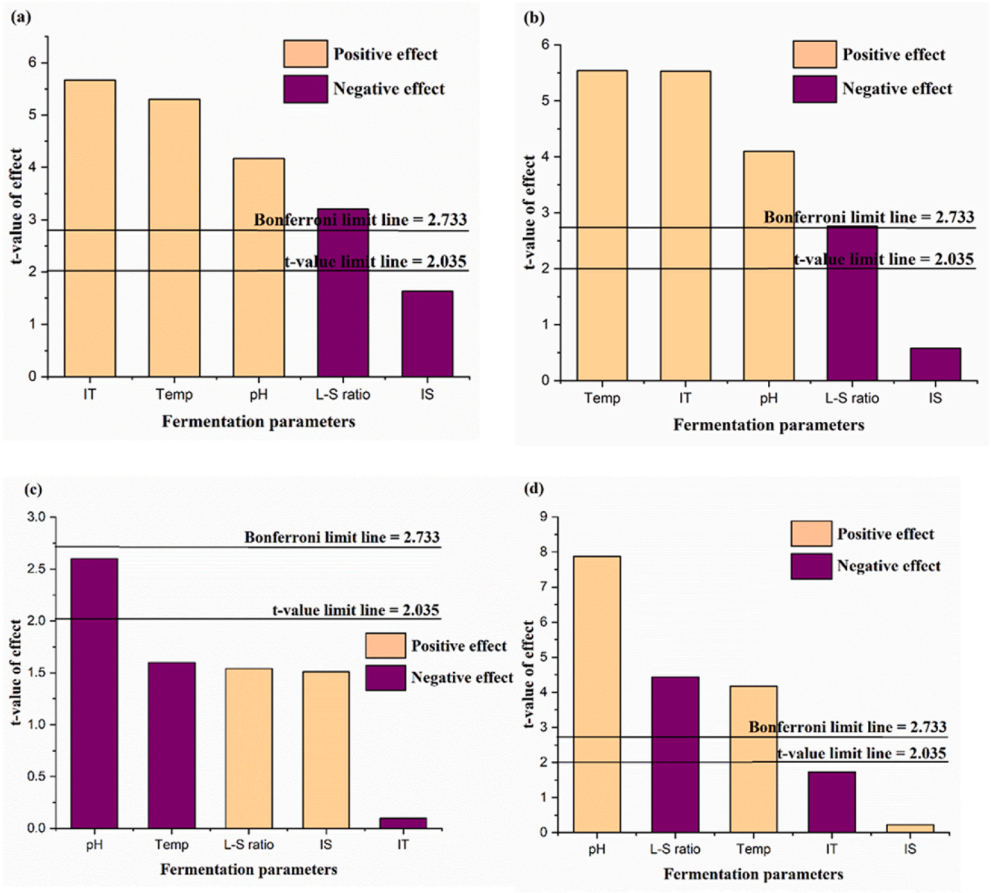

Fig. 1. Pareto chart for 5-*O*-caffeoylquinic acid yield (a), fermentation efficiency (b), total phenolic acids (c) and antioxidant activity (d) of LHFHS sample. Parameters having *t*-values greater than 2.035 (critical value) were regarded significant statistically.

3.2. Optimization of significant SSF conditions for 5-CQA extraction using Box-Behnken design

Optimization of SSF technique for 5-CQA extraction by selection of the best conditions is important to increase the 5-CQA yield. The significant factors (temperature, pH, incubation time and liquid-solid ratio) chosen from Plackett-Burman design screening analysis were considered for further optimization using response surface methodology. Response surface methodology is a group of mathematical and statistical techniques that optimizes conditions for an assured goal via establishment of a model from analysis of problems involving one or more responses of interest affected by several variables (Tang, Zhang, & Fang, 2015). The levels of the factors used for the optimization study were set based on the previous screening analysis. The experimental conditions and extraction yield from HS variety using four-factor-three-level Box-Behnken design were presented in Table 1. Response surface methodology explains the nature of data set and makes mathematical prediction. It saves time, sample use, and space, hence, more favourable relative to single-factor optimization (Lee et al., 2012). Multiple regression analysis was performed on the experimental data (Table 2) to evaluate for significance. The mathematical model for the SSF conditions optimization for extraction of 5-CQA from HS variety was achieved with second-order polynomial equation through investigation of the relationships between the independent (process) and the dependent (responses) variables.

3.3. Fitting models for extraction of 5-CQA from HS variety under SSF method

The experimental results obtained from Box-Behnken design analysis (Table 1) showed that temperature, pH, incubation time and liquid-solid ratio had significant influence on the 5-CQA yield of the LHFHS sample. The effect of the studied variables, parameter interactions, and arithmetic significance of the model was evaluated with analysis of variance. Table 2 displayed the *F*- and *p*-values of regression coefficients for the response variables. The quadratic polynomial model deduced from the highly significant *p*-values (<0.0001) of the models gave good estimates for the responses measured, indicating the fitness of the model. The R^2 values for 5-CQA yield, fermentation efficiency and antioxidant activity (0.9989, 0.9927 and 0.9996 respectively) affirmed it. In addition, the lack of fit values (*p*-value = 0.8588, 0.5151 and 0.2372) for the responses (5-CQA yield, fermentation efficiency and antioxidant activity respectively) were statistically not significant, confirmatory of model adequacy.

3.3.1. Influence of SSF parameters on 5-CQA yield of HS variety

Table 1 showed the 5-CQA yield obtained from LHFHS under SSF, which varied from 2.00 ± 0.06–11.41 ± 0.07 mg/g. The effect of SSF

Table 1
Box-Behnken design matrix with experimental design and data for the extraction of 5-CQA by SSF technique for one experimental block with *Lactobacillus helviticus*.

Run	Fermentation parameters (actual and coded values)				Response[c]		
	Temperature (°C)	pH	Incubation time (h)	Liquid-solid ratio	5-CQA yield (mg/g)	Fermentation efficiency (%)	Antioxidant activity (μmol AA eq/g dry sample)
	X_1	X_2	X_3	X_4			
1	40.00 (0)	5.00 (−1)	24.00 (0)	0.67 (+1)	9.40 ± 0.04	29.50 ± 0.18	35.77 ± 2.20
2	40.00 (0)	5.00 (−1)	24.00 (0)	0.25 (−1)	4.14 ± 0.02	17.32 ± 0.08	49.42 ± 1.51
3	50.00 (+1)	6.00 (0)	0.00 (−1)	0.46 (0)	4.12 ± 0.05	16.35 ± 0.21	46.55 ± 1.23
4	40.00 (0)	5.00 (−1)	48.00 (+1)	0.46 (0)	3.21 ± 0.09	16.80 ± 0.00	48.65 ± 1.42
5	50.00 (+1)	5.00 (−1)	24.00 (0)	0.46 (0)	5.33 ± 0.02	20.68 ± 0.07	38.51 ± 2.34
6	50.00 (+1)	6.00 (0)	24.00 (0)	0.67 (+1)	10.71 ± 0.03	30.81 ± 0.11	44.83 ± 1.37
7	30.00 (−1)	6.00 (0)	0.00 (−1)	0.46 (0)	2.00 ± 0.06	16.46 ± 0.25	22.19 ± 2.78
8	40.00 (0)	6.00 (0)	0.00 (−1)	0.25 (−1)	5.37 ± 0.02	21.56 ± 0.08	37.44 ± 1.81
9	30.00 (−1)	6.00 (0)	48.00 (+1)	0.46 (0)	5.46 ± 0.02	22.09 ± 0.07	48.95 ± 2.48
10	40.00 (0)	6.00 (0)	24.00 (0)	0.46 (0)	4.45 ± 0.01	18.17 ± 0.04	36.19 ± 2.89
11	40.00 (0)	6.00 (0)	24.00 (0)	0.46 (0)	4.17 ± 0.03	18.04 ± 0.11	36.48 ± 0.91
12	30.00 (−1)	5.00 (−1)	24.00 (0)	0.46 (0)	2.90 ± 0.05	16.02 ± 0.21	47.00 ± 1.42
13	30.00 (−1)	6.00 (0)	24.00 (0)	0.25 (−1)	6.13 ± 0.09	24.19 ± 0.36	48.21 ± 1.48
14	50.00 (+1)	6.00 (0)	48.00 (+1)	0.46 (0)	3.10 ± 0.01	16.96 ± 0.04	24.68 ± 2.18
15	40.00 (0)	6.00 (0)	0.00 (−1)	0.67 (+1)	6.38 ± 0.02	22.39 ± 0.08	29.63 ± 2.03
16	30.00 (−1)	7.00 (+1)	24.00 (0)	0.46 (0)	8.02 ± 0.03	27.00 ± 0.11	25.00 ± 2.14
17	40.00 (0)	5.00 (−1)	0.00 (−1)	0.46 (0)	2.35 ± 0.07	16.83 ± 0.28	36.94 ± 3.22
18	40.00 (0)	6.00 (0)	24.00 (0)	0.46 (0)	4.15 ± 0.07	19.00 ± 0.28	36.37 ± 1.30
19	40.00 (0)	6.00 (0)	48.00 (+1)	0.67 (+1)	10.96 ± 0.03	32.49 ± 0.11	37.29 ± 2.21
20	50.00 (+1)	7.00 (+1)	24.00 (0)	0.46 (0)	5.48 ± 0.04	19.33 ± 0.19	32.59 ± 2.49
21	40.00 (0)	6.00 (0)	48.00 (+1)	0.25 (−1)	3.00 ± 0.02	15.05 ± 0.07	35.90 ± 2.30
22	40.00 (0)	6.00 (0)	24.00 (0)	0.46 (0)	4.39 ± 0.05	18.05 ± 0.21	36.10 ± 2.94
23	40.00 (0)	7.00 (+1)	24.00 (0)	0.67 (+1)	11.41 ± 0.07	31.10 ± 0.31	32.33 ± 2.42
24	40.00 (0)	6.00 (0)	24.00 (0)	0.46 (0)	4.49 ± 0.08	17.24 ± 0.35	36.11 ± 4.54
25	50.00 (+1)	6.00 (0)	24.00 (0)	0.25 (−1)	4.47 ± 0.04	17.80 ± 0.18	24.60 ± 3.24
26	40.00 (0)	7.00 (+1)	48.00 (+1)	0.46 (0)	6.39 ± 0.03	25.13 ± 0.12	25.65 ± 3.69
27	30.00 (−1)	6.00 (0)	24.00 (0)	0.67 (+1)	8.91 ± 0.05	28.80 ± 0.23	22.29 ± 4.27
28	40.00 (0)	7.00 (+1)	24.00 (0)	0.25 (−1)	7.28 ± 0.03	26.10 ± 0.15	24.63 ± 4.56
29	40.00 (0)	7.00 (+1)	0.00 (−1)	0.46 (0)	4.56 ± 0.01	17.09 ± 0.04	31.91 ± 4.66

X_1 = Temperature; X_2 = pH; X_3 = Incubation time; X_4 = Liquid-solid ratio, [C]: Data were average values (x3).

conditions on 5-CQA yield from the LHFHS was shown in Fig. 2. On the basis of the *p*-values from the results, liquid-solid ratio was the noticeable most important parameter positively and significantly ($p < 0.0001$) influencing 5-CQA yield (Table 2). 5-CQA yield of the LHFHS sample increased with increasing liquid-solid ratio (to 0.67 maximum). The next obvious parameter was pH, which had significant positive effect on 5-CQA yield of LHFHS sample. Temperature, however, was positive but its effect was insignificant. Fig. 2c, obviously showed that the liquid-solid ratio–temperature interaction tremendously increased the 5-CQA yield of LHFHS sample positively. This implied that collectively increasing liquid-solid ratio and temperature increased the 5-CQA yield of the LHFHS sample. Though pH had lower positive *F*-value than that of liquid-solid ratio, its effect was significant on the 5-CQA yield.

The results showed that 5-CQA yield increased with increasing pH (Fig. 2a, d and e). Organic acids production and scale up are influenced by pH (Yazid, Barrena, Komilis, & Sánchez, 2017). pH influences microbial growth, proliferation and substrate colonization. It also enhances the efficiency of SSF, hence the observed increased yield of 5-CQA of LHFHS obtained with increasing pH. Relative to liquid-solid ratio and pH, incubation time had the lowest positive but significant effect on the 5-CQA yield. 5-CQA yield of the LHFHS sample increased with increasing incubation time. The interaction between incubation time and liquid-solid ratio positively increased the 5-CQA yield of LHFHS sample. Handa et al. (2019) reported similar effect of liquid-solid ratio in their study studies which was also on production of bioactive compounds under SSF.

The interactions between all the parameters influenced (negatively or positively) 5-CQA yield significantly. Thus, as incubation time and temperature increased, the 5-CQA of the LHFHS decreased (Fig. 2b). However, as liquid-solid ratio and incubation time, pH or temperature increased, the 5-CQA of the LHFHS also increased (Fig. 2; c, e and f). Similarly, as both pH and incubation time increased, the 5-CQA of the LHFHS increased (Fig. 2d). Also, as temperature decreased and pH increased, the 5-CQA of the LHFHS increased (Fig. 2a). *L. helviticus* possesses cinnamoyl esterase enzymes (temperature specific) which hydrolyze ester bonds resulting in the release of 5-CQA (Aguirre Santos, Schieber, & Weber, 2018). This suggests that the observed decrease in yield of the 5-CQA as a function of increases in incubation time and temperature, was due to inhibition of the activity of cinnamoyl esterase enzymes, whereas the increase in 5-CQA yield realized (as temperature decreased and pH increased) was as a result of the creation of optimum conditions for cinnamoyl esterase enzymes to efficiently hydrolyze the ester bonds of HS variety to release more 5-CQA. Increased liquid-solid ratio, incubation time and pH created optimum conditions for *L. helviticus* proliferation, resulting in degradation of the cell walls of the HS variety to release considerable quantity of 5-CQA into solution which

Table 2
Analysis of variance, regression analysis and optimal conditions for 5-CQA extraction from HS variety by SSF using *Lactobacillus helviticus*.

Source	5-CQA yield (mg/g)		Fermentation efficiency (%)		Antioxidant activity (μmol AA eq/g dry sample)	
	F-value	p-value	F-value	p-value	F-value	p-value
Model	870.13	<0.0001***	136.58	<0.0001***	2825.68	<0.0001***
Linear						
X_1 = A: Temperature	0.24	0.6336 ^{NS}	32.31	<0.0001***	5.68	0.0318*
X_2 = B: pH	1346.07	<0.0001***	165.70	<0.0001***	11397.43	<0.0001***
X_3 = C: Incubation time	290.13	<0.0001***	64.47	<0.0001***	435.76	<0.0001***
X_4 = D: Liquid-Solid ratio	4037.12	<0.0001***	570.53	<0.0001***	524.60	<0.0001***
Interactions						
AB	399.06	<0.0001***	92.39	<0.0001***	1247.62	<0.0001***
AC	324.25	<0.0001***	15.31	0.0016**	11410.88	<0.0001***
AD	193.41	<0.0001	42.88	<0.0001***	10276.70	<0.0001***
BC	15.20	0.0016**	39.58	<0.0001***	1558.14	<0.0001***
BD	20.63	0.0005***	31.33	<0.0001***	2199.41	<0.0001***
CD	780.36	<0.0001***	167.67	<0.0001***	408.40	<0.0001***
Quadratic						
A^2	34.15	<0.0001***	16.32	0.0012**	20.70	0.0005***
B^2	245.58	<0.0001***	52.16	<0.0001***	0.13	0.7284 ^{NS}
C^2	363.19	<0.0001***	20.63	0.0005***	16.15	0.0013**
D^2	3714.12	<0.0001***	585.76	<0.0001***	77.97	<0.0001***
Fitting statistics						
Lack of fit	0.45	0.8588 ^{NS}	1.08	0.5151 ^{NS}	2.17	0.2372 ^{NS}
R^2	0.9989		0.9927		0.9996	
Adjusted R^2	0.9977		0.9855		0.9993	
Predicted R^2	0.9956		0.9664		0.9982	
Adeq. Precision	105.274		37.401		166.000	
C.V. %	2.22		3.01		0.64	
Standard Dev.	0.12		0.64		0.23	
Optimization equations						

$$5-CQA\ yield\ (mg/g) = 4.33 - 0.018X_1 + 1.32X_2 + 0.61X_3 + 2.28X_4 - 1.24X_1X_2 - 1.12X_1X_3 + 0.87X_1X_4 + 0.24X_2X_3 - 0.28X_2X_4 + 1.74X_3X_4 + 0.29{X_1}^2 + 0.77{X_2}^2 - 0.93{X_3}^2 + 2.98{X_4}^2$$

$$Fermentation\ efficiency\ (\%) = 18.1 - 1.05X_1 + 2.38X_2 + 1.49X_3 + 4.42X_4 - 3.08X_1X_2 - 1.25X_1X_3 + 2.1X_1X_4 + 2.02X_2X_3 - 1.79X_2X_4 + 4.15X_3X_4 + 1.02{X_1}^2 + 1.82{X_2}^2 - 1.14{X_3}^2 + 6.09{X_4}^2$$

$$Antioxidant\ activity\ (\mu mol\ AA\ eq\ /g\ dry\ sample) = 36.25 - 0.16X_1 - 7.01X_2 + 1.37X_3 - 1.5X_4 + 4.02X_1X_2 - 12.16X_1X_3 + 11.54X_1X_4 - 4.49X_2X_3 + 5.34X_2X_4 + 2.3X_3X_4 - 0.41{X_1}^2 - 0.032{X_2}^2 - 0.36{X_3}^2 - 0.79{X_4}^2$$

*, ** and *** denote significance at $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively while NS denotes not significant.

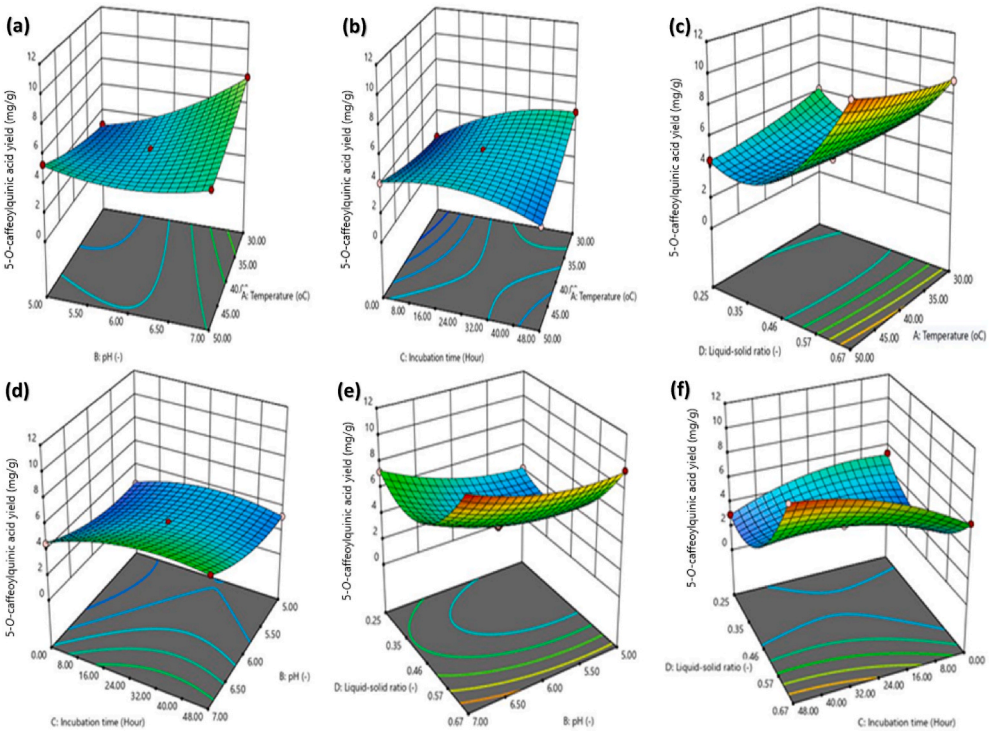

Fig. 2. Contour and response surface plots showing interactive influence of temperature, pH, incubation time and liquid-solid ratio on the 5-*O*-caffeoylquinic acid yield of LHFHS sample.

contributed to the observed increased 5-CQA yield of the LHFHS sample. All the quadratic terms of the model significantly influenced 5-CQA yield of the LHFHS sample. A second-degree quadratic equation generated from regression analysis was used to examine the association between the SSF parameters and response variables. The statistically insignificant ($p > 0.05$) term (temperature), was removed from the model to obtain a better fit model. The regression equation describing the effectiveness of SSF in coded variables for achieving the maximum 5-CQA yield of LHFHS sample was:

$$5-CQA\ (mg/g) = 4.33 + 1.32X_2 + 0.61X_3 + 2.28X_4 - 1.24X_1X_2 - 1.12X_1X_3 + 0.87X_1X_4 + 0.24X_2X_3 - 0.28X_2X_4 + 1.74X_3X_4 + 0.29X_1^2 + 0.77X_2^2 - 0.93X_3^2 + 2.98X_4^2 \quad (6)$$

3.3.2. *Influence of SSF parameters on fermentation efficiency of L. helviticus on HS variety*

From the results (Table 1), the obtained fermentation efficiency of the LHFHS sample using SSF was within the range of 15.05 ± 0.07 to 32.49 ± 0.11%. The maximum fermentation efficiency was attained at temperature of 40.00 °C, pH of 6.00, incubation time of 48.00 h, and liquid-solid ratio of 0.67. All the SSF parameters (temperature, pH, incubation time and liquid-solid ratio) investigated significantly influenced fermentation efficiency. However, only pH, incubation time and liquid-solid ratio had positive effect on the fermentation efficiency. Temperature exhibited a general decreasing effect on the fermentation efficiency, even though there was a slight increase after the mid-point (about 45.00 °C) (Fig. 3c). Thus, fermentation efficiency decreased with increased temperature (Fig. 3a and b). This was clearly shown by the perturbation plot. Temperature affects microbial growth. High temperatures kill microorganisms (inhibiting their activity) while optimum temperatures enhance microbial proliferation and thus their efficiency, leading to production of desired products. The inverse relationship between temperature and fermentation efficiency of *L. helviticus* observed in the present study could be that most of *L. helviticus* were killed as temperature increased. Handa et al. (2019) observed similar decreasing effect of temperature on bioactive compounds production using SSF.

Both the interactions between all the SSF parameters and their quadratic terms significantly influenced the fermentation efficiency of *L. helviticus* in the LHFHS sample (Table 2). Specifically, as liquid-solid ratio and temperature, pH or incubation time increased, the fermentation efficiency of *L. helviticus* also increased (Fig. 2; c, e and f). Likewise, as temperature decreased and pH or incubation time increased, the fermentation efficiency increased (Fig. 2; a and b). Also, as both pH and incubation time increased, the fermentation efficiency increased (Fig. 2d). Suggestive that optimum conditions for *L. helviticus* proliferation were attained when liquid-solid ratio and temperature, pH or incubation time increased (also as both pH and incubation time

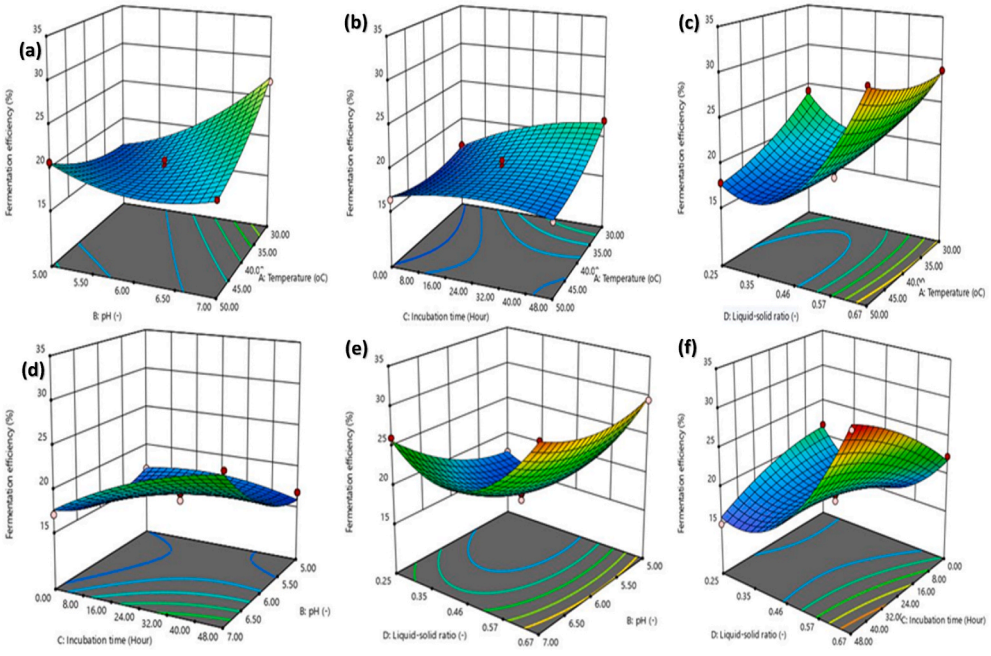

Fig. 3. Contour and response surface plots showing interactive influence of temperature, pH, incubation time and liquid-solid ratio on the fermentation efficiency of *L. helviticus* in LHFHS sample.

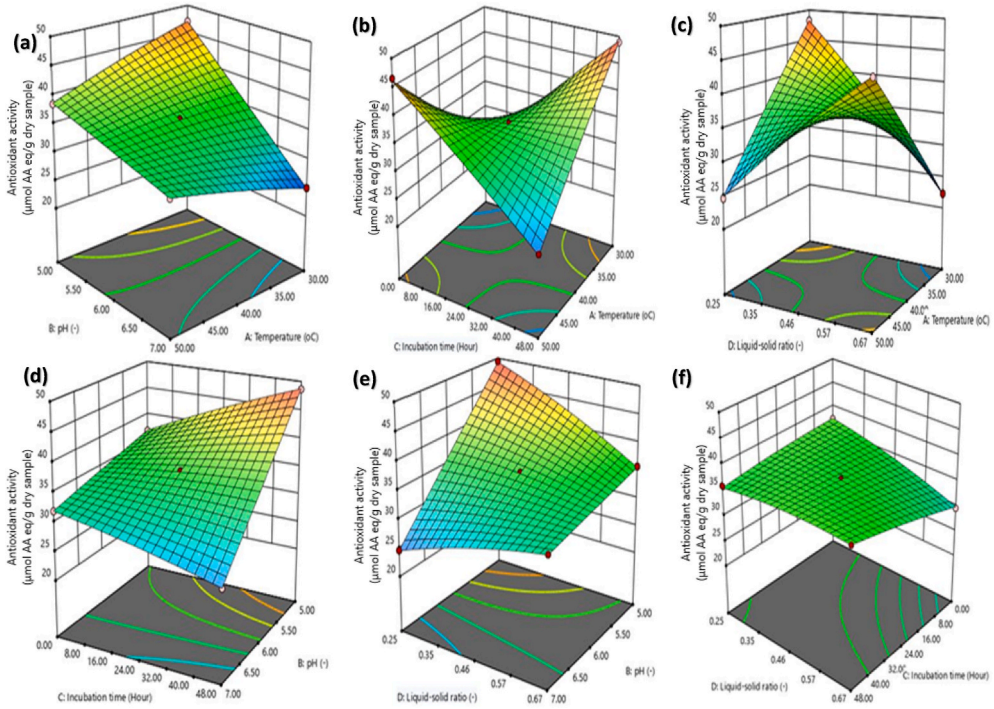

Fig. 4. Contour and response surface plots showing interactive influence of temperature, pH, incubation time and liquid-solid ratio on the antioxidant activity of LHFHS sample.

increased), which in turn increased the activity of *L. helviticus* resulting in the observed high fermentation efficiency. This scenario was same as, temperature decreased and pH or incubation time increased and resulted in similar result mentioned above. The predicting quadratic equation showing the effectiveness of the optimized SSF parameters in obtaining high fermentation efficiency of *L. helviticus* in the LHFHS sample, written in coded variables was:

$$\textit{Fermentation efficiency}\ (\%) = 18.1 - 1.05X_1 + 2.38X_2 + 1.49X_3 + 4.42X_4 - 3.08X_1X_2 - 1.25X_1X_3 + 2.1X_1X_4 + 2.02X_2X_3 - 1.79X_2X_4 + 4.15X_3X_4 + 1.02X_1^2 + 1.82X_2^2 - 1.14X_3^2 + 6.09X_4^2 \quad (7)$$

3.3.3. *Influence of SSF parameters on antioxidant activity of HS variety*

Auto-oxidation of food components is prevented by antioxidants. Antioxidants also neutralize the excess free radicals produced in the human body (Hur, Lee, Kim, Choi, & Kim, 2014). Numerous fermented products have high antioxidant activity, hence very useful in this regard. Considering that, fermentation of food materials is a valuable technology for improvement of the antioxidant activity of food products. In accordance with this, the antioxidative activity of LHFHS sample was evaluated with 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity method. The effect of four SSF parameters on the antioxidant activity of LHFHS sample was investigated. The effects of pH, incubation time and liquid-solid ratio on antioxidant activity of the LHFHS sample were found to be extremely significant while that of temperature was significant (Table 2). The results revealed pH to have the most significant effect on antioxidant activity but with a decreasing activity (Fig. 4a, d and e). The antioxidant activity of LHFHS sample steeply decreased as pH increased; confirmed by the perturbation plot. Adebo, Njobeh, Adebiyi, and Kayitesi (2018) reported similar results and attributed it to rearrangement of the phenolic structures caused by the acidic environment of the fermentation process as the pH increased, leading to phenolic compounds undergoing self-polymerization and/or interacting with other macromolecules like amino acids and starch, hence reducing their extractability. According to Hur et al. (2014), during fermentation, antioxidant activity is influenced by pH changes through changing of the phenolic compounds contents and structure. Similar results (decreased antioxidant activity at increased pH) were reported by Ruenroengklin et al. (2008) also.

Furthermore, all the interactive terms showed positive, extremely significant ($p < 0.0001$) effect on antioxidant activity. The results also found three quadratic terms out of four to exhibit significant effect on antioxidant activity. The quadratic effect of pH on antioxidant activity

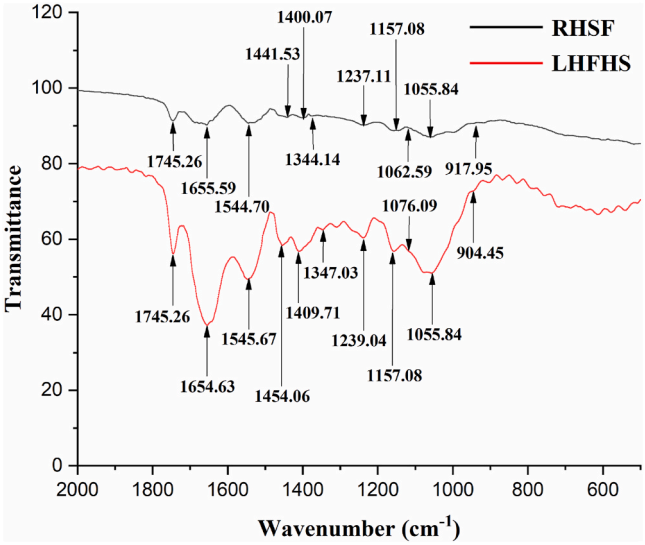

Fig. 5a. FTIR spectra of RHSF and LHFHS samples.

was not significant, though exhibited a positive influence. The antioxidant activity obtained for the LHFHS from the study (under the experimental SSF conditions) ranged from 22.19 ± 2.78 to 49.42 ± 1.51 μmol AA eq/g dry sample. The lowest antioxidant activity (22.19 ± 2.78 μmol AA eq/g dry sample) was obtained under temperature = 30.00 °C, pH = 6.00, incubation time = 0.00 h and liquid-solid ratio = 0.46 experimental SSF conditions, whilst the highest antioxidant activity (49.42 ± 1.51 μmol AA eq/g dry sample) was attained at temperature = 40.00 °C, pH = 5.00, incubation time = 24 h and liquid-solid ratio = 0.25 SSF conditions. This implied that at higher pH and liquid-solid ratio, and lower temperature and incubation time, SSF conditions may not be desirable for the production of LHFHS with highest antioxidant activity. The regression equation for explaining the effectiveness of the optimized SSF conditions for producing LHFHS with maximum antioxidant activity after the removal of insignificant term was written in coded variables as follows:

3.3.4. *Verification and validation of predictive model*

All the dependent variables/responses (5-CQA yield, fermentation efficiency and antioxidant activity) investigated were experimented

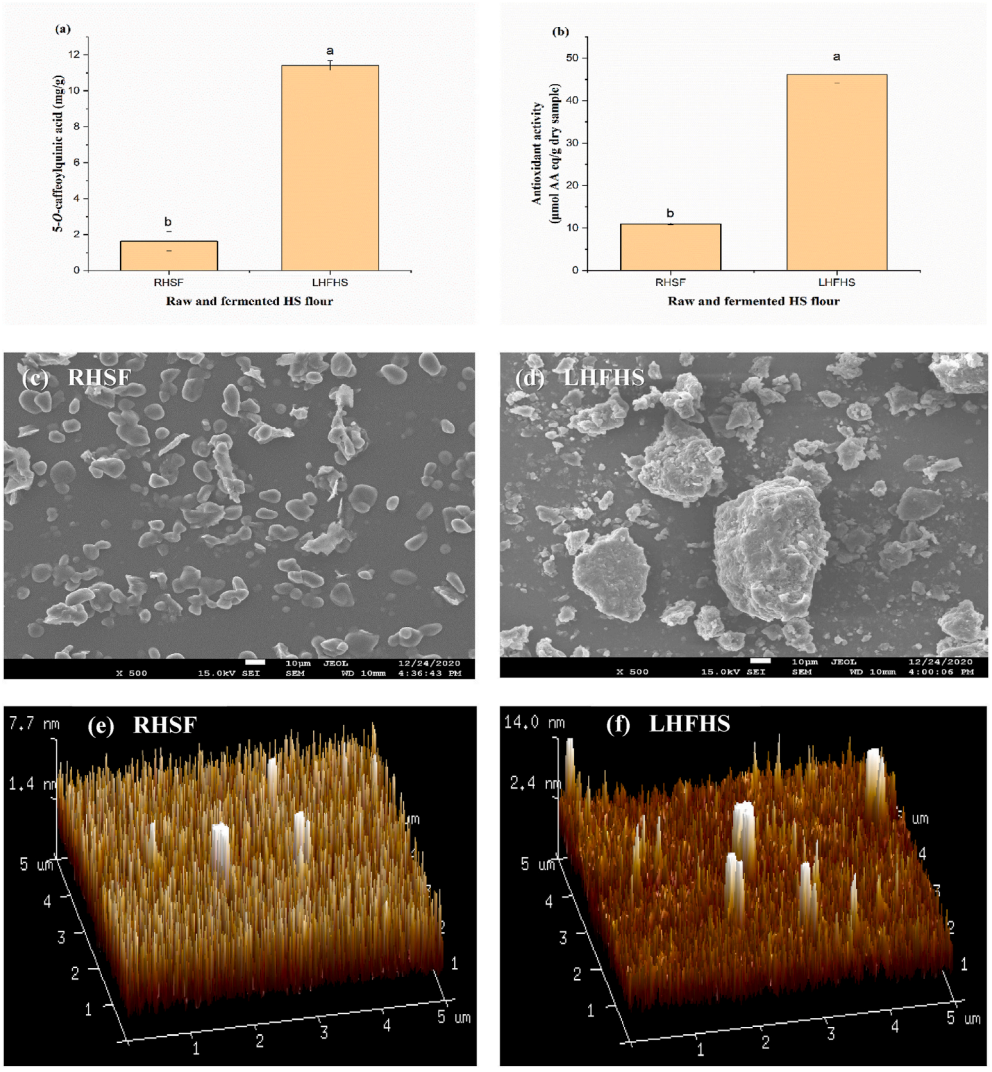

Fig. 5b. 5-*O*-caffeoylquinic acid yield (a), antioxidant activity (b), SEM and AFM micrographs of RHSF and LHFHS samples.

$$\text{Antioxidant activity } (\mu mol\ AA\ eq\ /\ g\ dry\ sample) = 36.25 - 0.16X_1 - 7.01X_2 + 1.37X_3 - 1.5X_4 + 4.02X_1X_2 - 12.16X_1X_3 + 11.54X_1X_4 - 4.49X_2X_3 + 5.34X_2X_4 + 2.3X_3X_4 - 0.41X_1^2 - 0.36X_3^2 - 0.79X_4^2 \quad (8)$$

under the predictive, optimized SSF conditions for 5-CQA extraction with increased yield and improved antioxidant activity to verify the model reliability. Temperature = 49.90 °C, pH = 7.00, incubation time = 25.81 h, and liquid-solid ratio = 0.67 were the optimized SSF conditions obtained for all the three responses. Under these optimized SSF conditions, both the predicted responses (5-CQA yield = 11.38 mg/g, fermentation efficiency = 30.48% and antioxidant activity = 46.12 μmol AA eq/g dry sample) and experimental responses (5-CQA yield = 11.41 ± 0.27 mg/g, fermentation efficiency = 30.49 ± 1.14% and antioxidant activity = 46.13 ± 1.94 μmol AA eq/g dry sample) were compared. The results of the experimental responses compared very well with that of the predicted responses. This proposed that the Box-Behnken design model obtained for the SSF of HS variety to extract 5-CQA with increased yield and improved antioxidant activity was efficient. Furthermore, the desirability of the model was 0.92. Jarpa-Parra et al. (2014) reported 0.6–0.8 (composite desirability) as a satisfactory value; thus, desirability index of 0.92 obtained is highly satisfactory.

3.4. Comparison of 5-CQA yield and antioxidant activity of fermented and unfermented HS variety

After the model validation, the experimental values of 5-CQA yield (11.41 ± 0.27 mg/g) and antioxidant activity (46.13 ± 1.94 μmol AA eq/g dry sample) obtained under the optimized SSF conditions were compared to that (5-CQA yield = 1.63 ± 0.53 mg/g and antioxidant activity = 10.98 ± 0.21 μmol AA eq/g dry sample) of the unfermented HS flour (RHSF). From the results, the optimized SSF conditions gave higher 5-CQA yield and antioxidant activity than the extraction from the unfermented sample (Fig. 5b; a and b). The high 5-CQA yield and antioxidant activity of the LHFHS sample obtained from the SSF model was due to the activities of *L. helviticus* through enzymes production which degraded the cell walls of the HS variety and broke the bonds between 5-CQA and other biomolecules (proteins, oligosaccharides, etc.). This resulted in the release of more free 5-CQA which gave the high 5-CQA yield with the improved antioxidant activity than the extraction from unfermented sample as observed. The results were in agreement with other studies which stated that hydroxycinnamic acids mostly exist in linked-form with cell walls (Santos da Silveira et al., 2019) and breaking of the bond between 5-CQA and oligosaccharides and/or polysaccharides through enzymatic degradation increases the content of free 5-CQA (Su, Cheng, Hsiao, Han, & Yu, 2018). Additionally, Taylor and Duodu (2015) stated that lactic acid bacteria metabolic activity during fermentation process involves various enzyme activities that influence the food chemical constituents, especially phenolic compounds, hence determining their fate in fermented products.

3.5. FTIR of the RHSF and LHFHS samples

The alterations in the chemical structure of HS variety after SSF were observed with FTIR. The structural changes of the samples (RHSF and LHFHS) were examined by the positional changes of the peaks of lignin, hemicellulose and cellulose. Consistent with the spectral trends, the sharp peaks observed at approximately 1056, 1157 and 1745 cm^{-1} (between 900 and 1800 cm^{-1}), corresponding to C–O stretching vibration of cellulose, hemicellulose and lignin (Loow et al., 2017), C═O ester groups stretching vibration of hemicellulose and C–O–C asymmetrical stretching vibration of hemicellulose and cellulose (Fakayode et al., 2020) respectively were same for both samples (Fig. 5a). The absorption peak at approximately 918 cm^{-1} in RHSF sample, shifted to approximately 904 cm^{-1} in LHFHS sample (between 800 and 1000 cm^{-1}) was mainly caused by the β-glycosidic linkage between cellulose and hemicellulose sugar units (Loow & Wu, 2018). The small sharp absorption peak of RHSF sample at approximately 1063 cm^{-1} indicating C–O stretching vibration of cellulose, hemicellulose and lignin (Loow et al., 2017) was intensified (shifted to approximately 1076 cm^{-1}) in LHFHS sample, suggesting that the lignin, hemicellulose and cellulose structures were exposed after the degradation of the cell walls of HS variety in the SSF. This possibly led to the release of more 5-CQA which gave the high content obtained. For the 1200–1400 cm^{-1} range, the absorption bands at approximately 1237 cm^{-1}, result of aryl–alkyl C–O–C ether bond of lignin (Fakayode et al., 2020) and 1344 cm^{-1}, result of C–H bending vibrations (Li, Wei, Xu, Xu, & He, 2018) in RHSF were shifted to 1239 and 1347 cm^{-1} in LHFHS, respectively. Regarding the 1400–1600 cm^{-1} range, the bands (in RHSF sample) at approximately 1400 cm^{-1} due to symmetrical CH_2– groups bending of cellulose (Loow & Wu, 2018), 1442 cm^{-1} due to C–H bending vibrations and 1545 cm^{-1} due to in-plane C═C aromatic vibration (Li et al., 2018) were shifted to approximately 1410, 1454 and 1546 cm^{-1} respectively in LHFHS sample. The alteration of the band from 1545 (in RHSF sample) to 1546 cm^{-1} (in LHFHS sample) justified that the SSF released significant quantities of phenolics (Loow et al., 2017). The absorption peak at approximately 1656 cm^{-1} in the interval of 1600–1700 cm^{-1} providing information on the O–H bending of absorbed water (Li et al., 2018) in RHSF sample was shifted to approximately 1655 cm^{-1} in LHFHS sample. This region was the most significant carbonyl absorption region as reported by Li et al. (2018). It was evident from the results that the SSF significantly impacted on the structure of the HS variety.

3.6. SEM of the RHSF and LHFHS samples

Comparison of the SEM images of RHSF and LHFS samples was done to examine morphological changes after SSF. The micrographs revealed that the granules of RHSF sample were spherical, small, fused (some), scattered and had consistent structure and smooth surface (Fig. 5b; c). However, in LHFHS sample, there was more noticeable granular structure degradation, changing it to a more loosened, irregular, rough surfaced with pits, and agglomerated granules (Fig. 5b; d). This suggested that there was degradation of starch and amino acids components, caused by the SSF, affirming the release of more, free 5-CQA, hence the high content with improved antioxidant activity obtained. The results conformed to the results of Adebo, Njobeh, Mulaba-Bafubiandi et al. (2018).

3.7. AFM of RHSF and LHFHS samples

AFM does not only image surfaces of biological structures in their native environment and oligomeric states, but visualizes conformational changes in the structures too. Based on this, it was applied in this study to examine the alterations in the topographic images of the samples (RHSF and LHFHS) after SSF. Fig. 5b; e and f depicted the AFM images of RHSF and LHFHS samples. Structural variations were observed between the two samples. The topographic image of RHSF particles was smaller in size and scattered with very few big ones. While that of LHFHS particles was loosed and big sized with irregular particles. The number of LHFHS particles was smaller than that of RHSF. Similarly, the particle heights of LHFHS sample were shorter than that of RHSF with minor

scattering and micropores. The results conformed to that reported by Dabbour et al. (2020). The results showed that the SSF significantly changed the structure of the HS variety, resulting in the extraction of the 5-CQA with increased yield and improved antioxidant activity.

4. Conclusion

In this study, a novel model for the extraction of 5-CQA with increased yield and enhanced antioxidant activity from HS variety was obtained. The model combined screening with quantitative multivariate calibration analysis based on SSF technique. Screening of SSF conditions by Plackett-Burman design showed that temperature, pH, incubation time and liquid-solid ratio contributed significantly to 5-CQA yield, fermentation efficiency and antioxidant activity. Box-Behnken design was successfully used to set the optimized SSF conditions; temperature = 49.90 °C, pH = 7.00, incubation time = 25.81 h and liquid-solid ratio = 0.67 under which maximum 5-CQA yield of 11.38 mg/g, fermentation efficiency of 30.49% and antioxidant activity of 46.12 μmol AA eq/g dry sample were obtained. The experimental and predicted data compared very well (desirability index, 0.92), making the model acceptable. Compared to extraction from unfermented HS variety, the extraction under the optimized SSF conditions gave higher 5-CQA yield with enhanced antioxidant activity. The study showed that extraction of 5-CQA with increased yield and enhanced antioxidant activity from HS variety under optimized SSF conditions was efficient, affirmed by Fourier transform infrared, Scanning electron microscopy and Atomic force microscopy micrographs. This study could help the food/pharmaceutical industry to produce cost-effective high 5-CQA yield with enhanced antioxidant activity from HS variety. Further studies on the bioactivities of 5-CQA from HS variety are therefore recommended.

CRediT authorship contribution statement

Nelson Dzidzorgbe Kwaku Akpabli-Tsigbe: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Yongkun Ma:** Supervision, Resources, Project administration, Funding acquisition. **John-Nelson Ekumah:** Investigation. **Juliet Osabutey:** Writing – review & editing. **Jie Hu:** Investigation. **Manqing Xu:** Investigation. **Nana Adwoa Nkuma Johnson:** Investigation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2021.111809.

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